

# **Myokine production and secretion in cultured human skeletal muscle cells**

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2007

## Acknowledgements

The present work was conducted from August 2006 to July 2007 at the Department of Nutrition, University of Oslo in the laboratory of professor Christian A. Drevon.

I would first of all like to thank my supervisor Christian A. Drevon for his enthusiasm for research, and his ability to motivate and support.

Special thanks to my co-supervisors Fred Haugen and Andreas Wensaas for always being available and for all the support during this year. I would also like to thank Anne Randi Enget for her technical support in the lab.

Terje Lund at Hormonlaboratory at Aker University Hospital is thanked for cooperation and performance of proteomics.

Further, thanks to my family for being encouraging and supporting regardless of my sometimes moody behavior during this year.

Finally, a special thanks to my lab partner, Katinka Stray, for numberless talks and good times shared. This year would not have been the same without you.

## Summary

**Introduction** During the last years it has become increasingly clear that different tissues in the body communicate with other tissues via hormonal signals synthesized and secreted to the blood as demonstrated in the last decade for the adipose tissue.

Skeletal muscle accounts for approximately 40 % of the body weight of a normal weight fit person. It is known that exercise offers protection against chronic disorders such as cardiovascular diseases, type 2 diabetes, dementia, and depression. It is unclear how contracting skeletal muscles mediate metabolic effects of benefits on health, which has been called the work stimulus, the work factor or the exercise factor. It has been shown that muscle fibres produce and release the cytokine IL-6 into the circulation during exercise. It is also possible that released myokines may explain how regular muscle activity can influence mood, performance, and cognitive function.

**Aims** The aims of this study were to investigate the release of peptides and proteins by human myotubes in culture and further study the effect of energy-carrying metabolites on the release.

**Methods** By analyzing the conditioned media by using proteomic analysis and ELISAs, we could measure the release of proteins from human myotubes in culture. We also used Brefeldin A (BFA) to further explore whether the release was due to secretion or due to leak because of cell damage.

**Results** The mean concentration of IL-6 from myotubes incubated in medium without BFA was 19 and 11 times higher than the concentration derived from myotubes incubated in medium with BFA, when incubating 3 and 6 hours, respectively. IL-6, IL-8, IL-7 and IL-13 were detected by Luminex Multiplex technology.

**Conclusion** We have during this work optimized several conditions important to consider when using myotubes as a model for identifying novel myokines. In addition to IL-6 we detected some potentially new myokines which are; IL-7, IL-8 and IL-13. Our studies support the theory that myotubes release proteins with possible hormonal functions, and that the release perhaps can be influenced by different energy carrying metabolites.

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# 1. Introduction

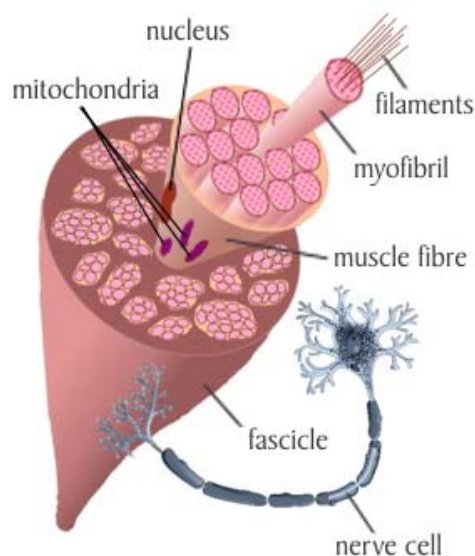
During the last years it has become increasingly clear that different tissues in the body communicate with other tissues via hormonal signals synthesized and secreted to the blood as demonstrated for the adipose tissue (1;2)

Several lines of evidence indicate that exercise promotes health (3-5). Some of the good consequences of regular exercise are greatly improved muscle tone, better posture, more efficient heart and lung function, less fatigue, and looking and feeling better. It is also known that exercise offers protection against chronic disorders such as cardiovascular diseases, type 2 diabetes, dementia and depression. The mechanisms for these health benefits are not fully understood. One of the possible explanations for the health benefit of exercise can be that regular muscle contraction mediates important messengers (6).

Muscle is a very specialized tissue that has both the ability to contract and the ability to conduct electrical impulses. Muscles are classified functionally as voluntary or involuntary, and structurally as either striated or smooth muscle cells. Muscles perform at least three essential functions in the body: contraction causing movement or providing posture, and heat production. Skeletal muscle accounts for approximately 40 % of the body weight of a normal weight fit person (7). Skeletal muscle is a type of striated muscle, usually attached to the skeleton. They generally contract voluntarily, via somatic nerve stimulation, although they can contract involuntarily via reflexes.

A skeletal muscle is composed of bundles of muscle fibers that generally extend the entire length of the muscle. They are called fibers, instead of cells, because of their threadlike shape. They can be 1 to 40 cm long but they have a diameter of only 10 to

100  $\mu\text{m}$ . Muscle cells contain network of tubules and sacs known as the sarcoplasmic reticulum a structure analogous but not identical to the endoplasmatic reticulum of other cells. Muscle fibers contain many mitochondria and, unlike most other cells, they have several nuclei.



**Illustration 1.**

Within each muscle cell are a myriad of tiny filaments encased in structures called myofibrils. The muscle fiber contains a lot of mitochondria (illustrated) and several nuclei (not illustrated). Illustration by Karen Wehrstein.

Skeletal muscle fibers can be classified in three types according to their structural and functional characteristics; slow, fast and intermediate fibers. Although all muscles contain a mix of all three fiber types, they have different proportions depending on the types of contraction that they most often perform.

Slow fibers are also called red fibers, because they contain a high concentration of myoglobin, the reddish pigment used by muscle cells to store oxygen. They are called slow fibers because their thick myofilaments are made of a type of myosin that reacts at a slow rate. Because red fibers contract slowly, they are usually able to produce



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ATP quickly enough to keep pace with the energy needs, and thereby avoid fatigue. This effect is enhanced by a larger number of mitochondria than other fibertypes and by the myoglobin-bound oxygen. Red fibers are well suited for chronically contractions and can hold the body upright for a long period without fatigue.

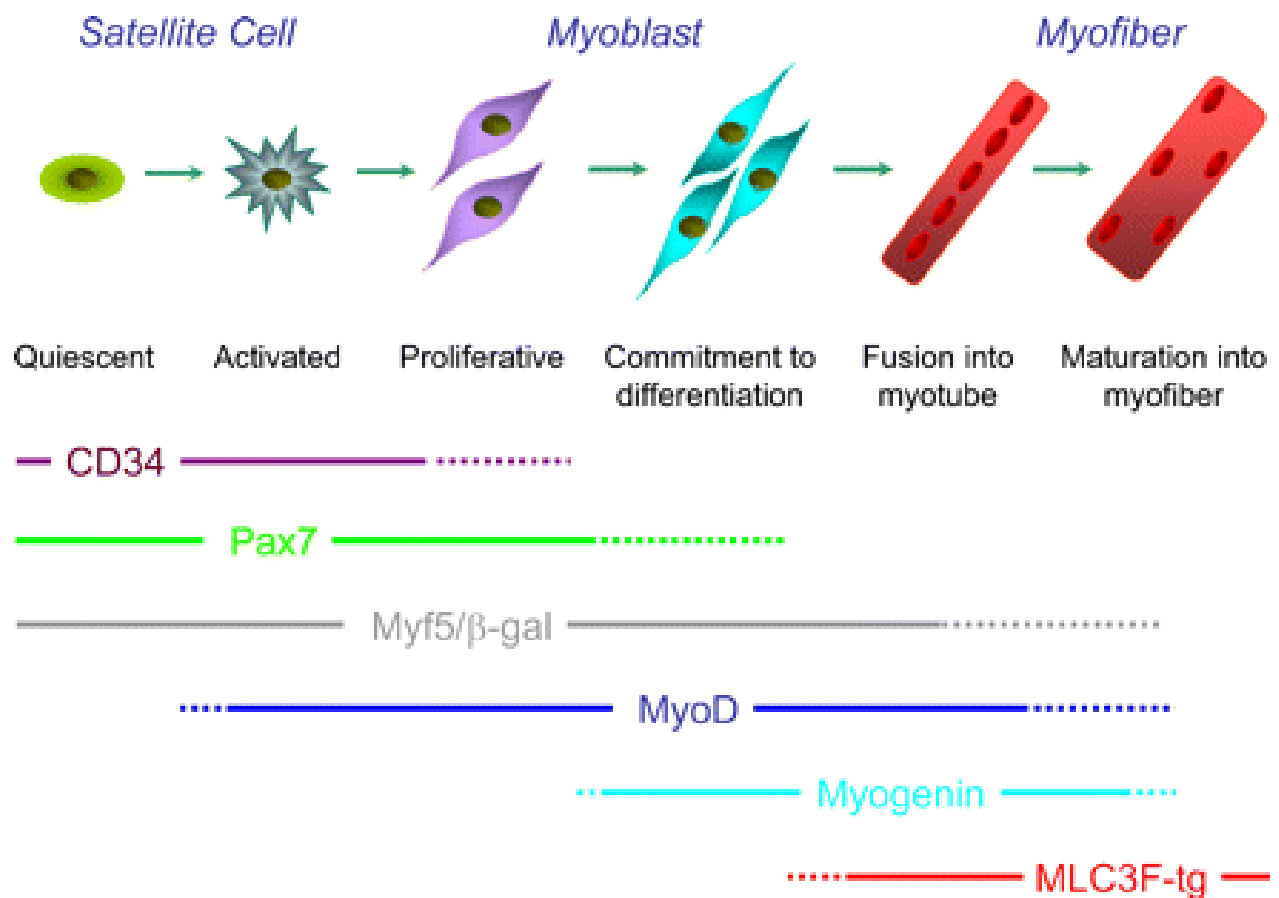
Fast fibers contain little myoglobin and contract much more rapidly than red fibers because they have a faster type of myosin. The fast fibers can rapidly be depleted of ATP during contraction and can not sustain contraction for a long time. This is because they quickly build up lactate due to increased glycolysis.

Intermediate fibers have characteristics in between the two extremes of fast and slow fibers. This type of fibers dominates in muscles that provide postural support and are occasionally required to generate rapid, powerful contractions (7).

Adult skeletal muscle is capable of self-repair in response to extreme training, injury or diseases such as muscular dystrophy. The ability for self-repair or regeneration is due to a population of satellite cells. The satellite cells are quiescent but become activated when a myofiber is injured. When myofiber repair or regeneration is needed the satellite cells activate, proliferate, and then withdraw from the cell cycle to form differentiated myotubes, and ultimately restore the skeletal muscle architecture (8).

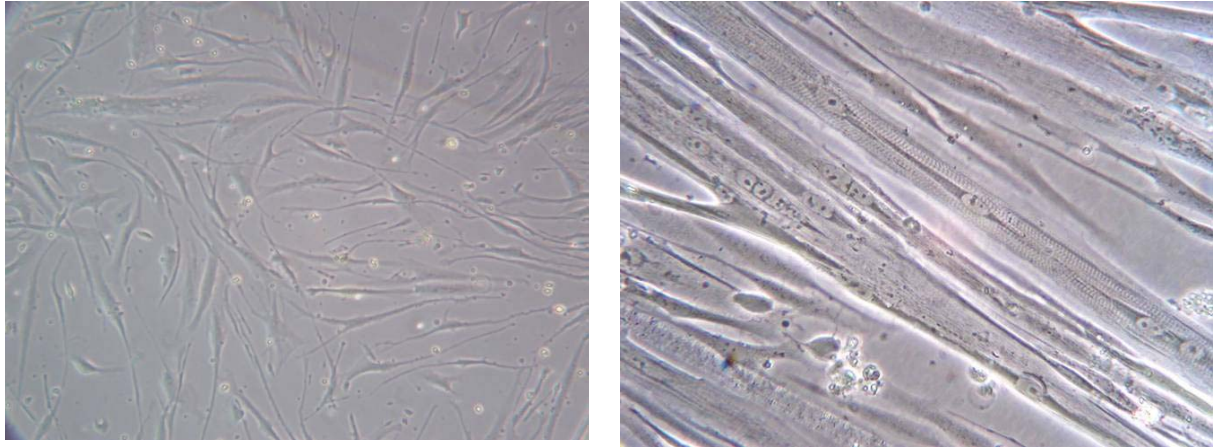
When the muscle fiber is not injured the satellite cell is quiescent and rests in an indentation in the muscle fiber. The satellite cells can be distinguished from the myonuclei by a surrounding basal lamina and more abundant heterochromatin. Satellite cells become activated and increase their cytoplasmic content when the fibre becomes injured. The cytoplasmic processes allow chemotaxis of the satellite cell along the myofiber (9).

Quiescent satellite cells express CD34, Pax7, and Myf5/ $\beta$ -gal. Pax7 is probably the most useful current marker for quiescent satellite cells due to the availability of a good antibody. When satellite cells are activated, they rapidly initiate MyoD expression, whereas myogenin later marks the commitment to differentiation.



**Illustration 2. A schematic presentation of satellite cell markers as they transit from quiescence to differentiation.** Satellite cells are quiescent in normal adult muscle and can be activated by, for example, muscle damage. Once activated, satellite cells divide to produce satellite cell-derived myoblasts that further proliferate, before committing to differentiation and fusing to form myotubes, which then mature into myofibers. CD34, Pax7, and Myf5/β-gal are expressed in quiescent satellite cells. Satellite cell activation is marked by the rapid onset of MyoD expression, whereas myogenin later marks the commitment to differentiation (10).

The satellite cells seeded out in culture are grown in a specialized medium and the change from one type of medium to another initiate the differentiation of the myoblasts.



**Picture 1. Human skeletal satellite cells seeded out in 12 well plate.** To the left; undifferentiated satellite cells. To the right; differentiated myotubes. Photo by Vigdis Aas.

Skeletal muscle atrophy can occur under many different conditions, including prolonged immobilization, denervation, cachexia, cushingoid conditions or with advanced age. The word “cachexia” is derived from the Greek “kakos” meaning “bad” and “hexis” meaning “condition”. Cachexia is the cytokine-associated wasting of protein and energy stores. Systemic inflammation mediated through cell injury or activation of the immune system triggers an inflammatory response. Persons with cachexia lose roughly equal amounts of fat and fat-free mass, while maintaining extracellular water and intracellular potassium. The loss of fat-free mass is mainly from the skeletal muscle. Cachexia due to cancer may deplete up to 80% of their muscle mass (11). The loss of skeletal muscle mass is due to a combination of reduced protein synthesis and increased protein degradation, the latter being the major factor (11).

In addition to the effect of cytokines on skeletal muscle, cytokines act in the hypothalamus to cause an imbalance between the orexigenic and anorexigenic regulatory pathways. In anorexia-cachexia syndrome, the peripheral signals for an

energy deficit reaching the hypothalamus fail to produce a response, which propagates the cachectic process. Despite a falling energy intake, patients with cachexia frequently show elevated resting energy expenditure as a result of increased Cori cycle activity, glucose and triglyceride-fatty acid cycling and gluconeogenesis. A number of cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukins 1 and 6 (IL-1 and IL-6), interferon  $\gamma$  (IFN- $\gamma$ ), and leukaemia-inhibitory factor (LIF), have been proposed as mediators of the cachectic process (12). IL-6 has the potential to act as a cachectic factor. In an in vitro study (13) using C2C12 myotubes, incubation of the cells to recombinant human IL-6, shortened the half-life of long-lived proteins and increased the proteolytic activity. This suggests that IL-6 is capable of up-regulating protein degradation. The results indicate that IL-6 may be active in the cachectic process. However, IL-6 probably does not act alone but may either induce or act in synergy with other cachectic factors (12).

Muscular dystrophies are inherited disorders characterized by muscle degeneration and associated progressive wasting and weakness. The most common form is Duchenne muscular dystrophy (DMD), affecting 1/3500 newborn males. It is caused by the dystrophin gene encoding a large protein that is crucial for the assembly of a cell surface complex connecting the muscle fibre cytoskeleton to the overlying basal lamina. In the absence of dystrophin, muscle fibres undergo repeated cycles of degeneration accompanied by regeneration involving extensive proliferation of muscle precursor cells, but also a chronic inflammatory response and cytokine production. This inflammation is associated with synthesis of extracellular matrix (ECM) molecules. Excessive accumulation of EMC in the interstitial space was the basis of the original description of this disease as myosclerosis (7).

Se-Jin Lee found in 1997 that the strong Belgian Blue and Piedmontese cattle strains had a defective myostatin gene; these strains have been produced through breeding. The mutated myostatin led to abnormal muscle growth (14;15). Myostatin (growth differentiation factor-8) is a secreted growth factor belonging to the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily that acts as a negative regulator of skeletal

muscle mass. Human myostatin consists of two identical subunits with 110 amino acid residues and has a total molecular weight of 25.0 kDa. A number of natural, inactivating mutations have been identified in cattle, mouse, sheep and human, all leading to a hypermuscular phenotype. The biological relevance of myostatin was also addressed in mice by gene inactivation, overexpression of normal or dominant-negative forms and systemic administration of inactivating antibody or myostatin protein. From these studies and many others carried out in cultured cells, the general conclusion is that myostatin regulates the final number of muscle fibers during development by blocking the proliferation and differentiation of myoblasts and also regulates the postnatal muscle-fiber size by maintaining the satellite cells in a quiescent state and inhibiting protein synthesis. Considering its effect on muscle growth, inhibition of myostatin has a tremendous potential as a therapeutic approach in muscle-degenerative and wasting conditions, such as muscular dystrophies and cachexia. (16;17)

## 1.1 Myokines

In vivo studies have shown that muscle tissue release peptides and proteins during exercise (18). In line with that adipokines have been suggested as a term to cover proteins that are produced and secreted by adipocytes, we suggest that the term “myokines” should be restricted as a collective term to describe proteins that are produced and released by muscle fibres per se.

The cytokine IL-6 is produced and released by muscle fibres into the circulation during exercise (18). Plasma levels of IL-6 increase exponentially up to 100-fold in relation to an acute bout of exercise. The size of the response depends on the intensity, duration, and the mode of exercise. The IL-6 transcription rate is markedly enhanced when glycogen levels are low (19).

Physical exercise is associated with a systemic cytokine response comparable with the levels observed during severe infections. One important difference is that the levels

of TNF- $\alpha$  and IL-1 $\beta$  are increased for a short period (minutes) if present at all when concentric exercise without muscle damage is performed. This indicates that in non-traumatic exercise models, the cytokine cascade differs markedly from the classical acute-phase response studied during infections (6;18).

IL-6 is an important pleiotropic cytokine with both pro- and anti-inflammatory properties. Increased circulating levels of IL-6 have been reported in severely ill patients suffering from injury, sepsis, cachexia, or cancer.(20). Muscle-derived IL-6 is considered to possess a central role in anti-inflammatory activities and health beneficial effects in relation to physical activity (6).

IL-6 is a member of a family of cytokines that consists of leukaemia inhibitory factor, IL-11, ciliary neurotropic factor, oncostatin M, and cardiotrophin 1 (21). Their membership is based on similarities in helical protein structure and a shared receptor subunit (22). IL-6 is a variably glycosylated protein with a molecular mass of 22–27 kDa depending on the cellular source and amount of post-translational modification. It is synthesized as a precursor protein of 212 amino acids (aa), with a 28 aa signal sequence and a 184 aa mature segment (23). IL-6 is produced by many different cells, but the main sources in vivo are stimulated monocytes/macrophages, fibroblasts, and vascular endothelial cells, indicative of its role in the modulation of the immune system. Other cells known to express IL-6 include keratinocytes, osteoblasts, T cells, B cells, neutrophils, eosinophils, mast cells, smooth muscle cells (24) in addition to skeletal muscle cells (25) Several studies have shown that adipose tissue also produces and secretes IL-6, and that 10-35% of the body basal circulating IL-6 is derived from adipose tissue (20).

IL-6 increases glucose metabolism in resting human skeletal muscle, as well as increasing transport of glucose into skeletal muscle, glucose incorporation into glycogen, and glucose oxidation (14). AMP-activated protein kinase (AMPK) is a fuel-sensing enzyme that is activated by changes in the energy status of a cell and by exposure to hormones as adiponectin and leptin, and catecholamines. Once activated, AMPK stimulates a variety of processes that increase ATP generation including fatty

acid oxidation and glucose transport in skeletal muscle cells (26). Data demonstrate that exercise activates transcription of the IL-6 gene in working skeletal muscle, a response that is dramatically enhanced when glycogen levels are low (27). Both IL-6 production and AMPK activity increase to the intensity and duration of exercise and are further enhanced when glycogen levels are low (26).

Proteins measured in plasma from working skeletal muscles can derive from muscle cells, either by leak or secretion, or from other kinds of cells in the muscle tissue e.g. macrophages. Studies have shown that proteins can derive from both skeletal muscle tissue and from muscle cells in culture (18;20;28). Proteins in plasma that are synthesized in skeletal muscles can not unconditionally be classified as myokines because they can be derived from cells other than muscle fibres. Another study by De Rossi et al. indicated that IL-8 was released by human myotubes in culture (29), but they did not show whether this release was due secretion or leakage from damaged cells.

In our study we want to find out more about the release and secretion of proteins from myotubes in culture. This will give us more information about myokines because in culture we know that the proteins we measure are derived from the muscle cells, and we can determine whether the release is due leak or secretion.

## 1.2 Aims

### **1.2.1 Study release of peptides and proteins by human myotubes in culture**

The first object of this study was to investigate if we could detect proteins released from cultured human myotubes in our model.

### **1.2.2 Study the effect of energy-carrying metabolites on the release of proteins from human myotubes in culture**

The second object was to incubate myotubes with energy-carrying nutrients and examine how this will influence the release of myokines.



## 2. Materials and methods

### 2.1 Materials

Dulbecco's modified eagle medium with L-Glutamine (DMEM), 10 % foetal calf serum (FCS) and penicillin-streptomycin 5000IU/mL - 5mg/mL was obtained from Sigma Chem, St.Lois, MO, USA. Ultrosor G was obtained from BioSeptra, CIPHERGEN Biosystem Inc, Paris, France. Extracellular matrix gel (ECM), essentially fatty acid free bovine serum albumin, bovine serum albumin (used for protein standard) Sigma Chem. Co, palmitic acid (PA), oleic acid (OA), eicosapentaic acid (EPA), DHA was obtained from Nu-Chek prep, Inc, Elysian, MN, USA. <sup>14</sup>C labelled BSA was delivered by Perkin Elmer, Waltham, Massachusetts 02451 USA. Insulin Actrapid was obtained from Novo Nordisk, Bagsværd, Denmark. Trizol reagent was purchased from Invitrogen, Carlsbad, CA, USA. Bio-Rad protein assay dye reagent concentrate was obtained from Bio-Rad Laboratories, CA, USA. 12-well plates were obtained from Corning, NY, USA. Total protein was measured with Titertek Multiskan, Labsystems Oy, Helsinki, Finland. Cell Bind Flasks Corning, NY, USA. DMEM w/o glucose, phenol red, L-Glutamine, Sigma Chem. Co and IL-6 immunoassay, came from R&D Systems, MN, USA. Multiplex Immunoassay was from Lincoplex, St. Charles. Missouri, USA, and glucose was delivered by Sigma Chem. Co, the LDH-kit was delivered by Roche Diagnostics, Roche Applies Science, Mannheim, Germany, whereas RD 193058100 Human Myostatin ELISA came from BioVendor. For protein assay, the BC Assay protein kit was obtained from Uptima Interchim, Montluçon, France.

A cell bank of satellite cells was established from muscle biopsy samples taken from musculus vastus lateralis or m. obliquus internus abdominis from healthy volunteers, aged  $24.7 \pm 0.7$  years with body mass index  $23.6 \pm 1.1$  kg/m<sup>2</sup>. The biopsies were

obtained with informed consent and approval by the National Committee for Research Ethics (Oslo, Norway). Muscle cell cultures free of fibroblasts were established by the method of Henry et al. with minor modifications. The cells were cultured in a humidified 5% CO<sub>2</sub> atmosphere at 37°C, and medium was changed every 2–3 days.

### **Medium 1 (M1) for seeding**

500 mL DMEM with low glucose (SIGMA, #D6046), 50 mL FCS (Foetal Calf Serum (SIGMA, #F7524), 5 mL L-glutamine (SIGMA, #G7513, 100 mL), 5 mL Penicillin/Streptomycin (GIBCO BRL, #15140-114, 100 mL)

### **M2 for proliferation**

500 mL DMEM with low glucose (SIGMA, #D6046), 10 mL FCS (Foetal Calf Serum (SIGMA, #F7524), 10 mL Ultrosor G, 5 mL L-glutamine (SIGMA, #G7513, 100 mL, 5 mL Penicillin/Streptomycin (GIBCO BRL, #15140-114, 100 mL)

### **M3 for differentiation**

500 mL DMEM with low glucose (SIGMA, #D6046), 10 mL FCS (Foetal Calf Serum (SIGMA, #F7524), 5 mL L-glutamine (SIGMA, #G7513, 100 mL), 5 mL Penicillin/Streptomycin (GIBCO BRL, #15140-114, 100 mL, 25 pmol Insulin ( $\Rightarrow$ 21  $\mu$ L of 1000 x diluted Insulin Actrapid<sup>®</sup>, Novo Nordisk, 0,6 mM)

Ultrosor-G (Bio Serpa, #P/N 15950-017) should be re-constituted by adding of 20 mL MQW to the container. Wait for 15 minutes, and transfer 10 mL to M2-medium. The rest (10 mL) is frozen (-20 °C) for further use.

## **2.2 Methods**

To study the in vitro secretion of myokines from human skeletal muscle cells, we had to make a model appropriate for our hypothesis. We performed a few experiments before we selected a model that would function for both analysis in the laboratory at

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UiO and for the analysis at the Hormonlaboratorium at Aker University hospital. Earlier experiments performed by Vigdis Aas and colleges indicated that it was important to remove as much protein from serum in the media as possible before the analysis at Aker University Hospital. This was necessary to reduce the background noise from FCS proteins (mainly BSA) during 2D-gel electrophoresis. Thus, to find possible proteins derived from myotubes in the supernatant, albumin and other proteins from the serum in the medium had to be removed from the supernatant before the samples could be analyzed at Aker by 2D-gel electrophoreses. To remove serumprotein we had to decide the optimal numbers of washes each well should receive. Some other things we had to find out were how many days the cells should be differentiated before experiments were performed, how long time the cells would stay viable without serum, at what concentrations of the different components given to the myotubes, and how we could explore whether the cells were leaking or secreting the proteins in media.

We performed several experiments to find answers to the questions above but also to learn the techniques. The differentiation medium contained abundant amounts of exogenous serum-derived proteins that had to be removed before analysis of myokine secretion could be performed. Especially albumin was potentially troublesome because it would generate a relatively high background if not properly removed by washing. Thus, it was very important to evaluate the washing procedure with respect to the removal of exogenous proteins. Extensive washing can be harmful to the cells, and therefore the aim was to find the number of wash-cycles that sufficiently removed the albumin without significantly reducing cell viability.

### **2.2.1 Seeding satellite cells**

The muscle cells used in this model were satellite cells isolated from biopsy samples from m. obliquus internus abdominis or m. vastus lateralis. The satellite cells had to

be treated in a special manner to manage to grow and differentiate and we followed the procedure as follows;

#### Procedure

1. 1 vial containing the satellite cells (approximately 1.5 million cells) was picked up from the tank containing liquid nitrogen.
2. The suspension was thawed in 37 ° C until only a tiny piece of ice was left.
3. In a LAF bench the cell suspension was added to a 50 mL tube containing 9 mL or 15 mL prewarmed M1 depending on the format used (12 well plate or 75 cm<sup>2</sup> flask). This was performed to avoid the cells to be exposed to the high concentration of DMSO which is poisonous for the cells when they are not frozen. The cell suspension was well mixed with M1 by a pipette.
4. The cells were seeded with 750 ul medium in each well in the 12 well plate and 15 mL in the 75 cm<sup>2</sup> flask and incubated in 37°C and 5% CO<sub>2</sub> over night.
5. The day after the medium was changed from M1 to 1 mL M2. M2 was changed every second or third day.
6. When the cells had reached 80% confluence the medium was changed to either 1 mL M3 for the 12 well plate or 15 mL for the flask. When changing medium to M3 at this point the cells differentiated into multinucleated myotubes. The M3-medium was changed every second or third day.
7. The experiments were performed either before M3-medium were given (0-days differentiating cells) up to 5-days differentiated cells. Different substances were added to either M3 or DMEM according to the experiment performed. The cells were visually inspected under microscope during growth, differentiation and experiments.

### **2.2.2 Monitoring cell damage by LDH activity**

The myotubes were exposed to various potentially harmful substances in the different experiments. To monitor their well being we used Cytotoxicity Detection Kit that measures how much of the intracellular enzyme lactate dehydrogenase (LDH) is leaking through the membrane. This can be used as a general marker on whether the cells are doing all right or not.

Protocol:

1. After the media, which the myotubes had incubated in, were centrifuged on 3000 rpm for 5 min, and the muscle cells lysated in 100-1000 ul 2% Triton X-100, 20 ul and 25 ul from the lysate and supernatants, respectively were transferred to a flat bottom 96-well plate.
2. To each well 25-30 ul milliQ water and 50 ul LDH reagents were added
3. The 96-well plate incubated in 37°C for 15 minutes
4. The absorbance was measured by a spectrophotometer (Titertec)
5. Blank background samples consisting of either only DMEM or lysis-solution were measured in parallel and the value were subtracted from the respective values from the samples

### **2.2.3 Protein quantification of the lysates**

BC (bicinchoninic acid) Assay and Bio-Rad Assay were used to determine the amount of protein in samples.

The BC-reagent reduces  $\text{Cu}^{2+}$  in alkaline solutions to  $\text{Cu}^{+}$  and two molecules of BCA interact with each molecule of  $\text{Cu}^{+}$  forming a water soluble red complex.

The Bio-Rad Protein Assay is a dye-binding assay in which a differential colour change occurs in response to the concentration of protein. Coomassie blue dye binds to primarily basic amino acid residues, especially arginine.

Protocol:

Cell lysates were homogenised and 25 ul of each homogenate was transferred to separate wells on a flat bottom 96-wells plate.

Protein standards made from BSA by serial dilution were likewise added to separate wells.

Each well was added 200 ul reconstituted coomassie reagent or BC-reagent, and the plate was then incubated at room temperature (5 or 30 min for Coomassie and BC-reagent, respectively).

The absorbance values were determined by a spectrophotometer (Titertec) at either 590 nm (Coomassie) or 490 nm (BC).

Sample protein concentrations were determined by plotting the absorbance values into the linear function obtained from the protein standards.

#### **2.2.4 IL-6 measurement**

ELISA is a sensitive immunoassay that uses an enzyme linked to an antibody as a marker for the detection of a specific protein.

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate.

Standards and samples were pipetted into the wells, and any IL-6 present was bound by the immobilized antibody.

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The samples were compared against the standards curve to determine the amount of IL-6 present. Results were expressed in pg/mL. The lower detection limit was 0.07 pg/mL.

### **Protocol**

1. 100 ul Assay Diluent were added to the wells
2. 200 ul of Standard, sample or control were added per well
3. The wells were washed by a washing procedure as follows;
  - a. Removing liquid from the wells by inverting the plate and decanting the contents.
  - b. Removing excess liquid by grasping the plate firmly and smartly rapping the plate inverted on a clean paper towel at least 5 times.
  - c. Filling each well with 400 ul of Wash Buffer using a multi-channel pipette
  - d. Removing liquid from the wells by inverting the plate and decanting the contents.
  - e. Repeating steps b, c, and d 3 times for a total of 4 washes. After the last wash, smartly rap the plate inverted on a clean paper towel at least 10 times to remove excess Wash Buffer.
4. 200 ul of GM-CSF Conjugate were added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature.
5. The wash steps above were repeated.
6. 50 ul of Substrate Solution were added to each well. The plate was covered with a new adhesive strip and incubated for 1 hour at room temperature.

7. 50 ul of Amplifier Solution were added to each well and covered with a new adhesive strip and incubate for 30 minutes at room temperature. Addition of Amplifier Solution initiated colour development.
8. 50 ul of Stop Solution were added to each well. Addition of Stop Solution does not affect the color in the wells.
9. The optical density of each well was determined within 30 minute, using a microplate reader set to 490 nm.

### **2.2.5 Brefeldin A (BFA) was utilized to explore protein secretion from myotubes**

BFA is a lactone antibiotic produced by fungal organisms. BFA interferes with protein transport from endoplasmatic reticulum to Golgi apparatus and leads to accumulation of proteins inside ER. In mammalian cells BFA inhibits activation of a GTPase which recruits coat proteins to intracellular membranes and is therefore involved in the formation of transport vesicles. Because BFA inhibits protein transport and secretion of proteins, we can get an indication on whether the proteins found in the media is due to leak or secretion.

The myotubes were added incubation medium with or without 1 ug/mL BFA. By comparing the amount of protein measured in the media from the two treatments we can explore the secretion of different peptides and proteins derived from myotubes.

### **2.2.6 Cytokine measurements**

High sensitivity human cytokine multiplex immunoassay kit may be used for analyses of many cytokines in culture supernatants simultaneously. The cytokines



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tested were IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IFN $\gamma$ , GM-CSF and TNF $\alpha$ .

### Procedure

1. The filter was pre-wet by pipetting 200  $\mu$ l of 1X Wash Buffer into each well of the microtiter plate
2. The plate was sealed and put on a plate shaker for 10 minutes at room temperature.
3. Wash Buffer was removed by vacuum.
4. The bead bottle was sonicated for 30 seconds and vortexed for 1 minute.
5. 25  $\mu$ l of the mixed beads were added to each well.
6. Liquid was removed from the wells by vacuum.
7. 50  $\mu$ l Assay Buffer were added to the 0 Standard and the sample wells.
8. 50  $\mu$ l of each Standard and Control were added to appropriate wells.
9. 50  $\mu$ l matrix solution were added to the Background, Standards, and Control wells.
10. The plate was covered with aluminium foil and incubated with agitation on a plate shaker overnight (19 hr)
11. The fluid was removed by vacuum aspiration.
12. The plate was washed with 200  $\mu$ l Wash Buffer 2 times. The removing of Wash Buffer was performed by vacuum.
13. 50  $\mu$ l of Detection Antibody Cocktail were added each well.
14. The plate was sealed with aluminium foil, and incubated on a plate shaker for 1 hour at room temperature.

15. 50 ul Streptavidin-Phycoerythrin were added each well.
16. The plate was covered with aluminium foil, and incubated with agitation on a shaker for 30 minutes at room temperature.
17. The content was removed by vacuum.
18. The plate was washed with 200 ul Wash Buffer two times and the Wash Buffer was removed by vacuum.
19. 100 ul of Sheat Fluid were added each well. The plate was covered with aluminium foil and resuspended the beads on plate shaker for 5 minutes.
20. The plate was read on Luminex Instrument.

## **2.2.7 Experiments**

### *Experiment 1*

#### **Cells incubated with $^{14}\text{C}$ -bovine serum albumin to measure the amount of protein removed during washing**

$^{14}\text{C}$ -bovine serum albumin ( $^{14}\text{C}$ -BSA) was used as a marker for macromolecules. A known concentration of  $^{14}\text{C}$ -BSA, which can be detected by scintillation counting, was added to the medium and the amount of  $^{14}\text{C}$ -BSA was monitored. The amount of  $^{14}\text{C}$ -BSA removed can be compared with the amount of the total of proteins removed.

### *Experiment 1a*

#### **Protocol 1a**

1. Myotubes were seeded and differentiated in three 12 well plates as described above.

2. After the myotubes were differentiated for four days the wells were added 1 mL radiolabelled medium and incubated in 37° C, in 5 % CO<sub>2</sub> for 24 hours. The concentration of <sup>14</sup>C-BSA in the medium (M3) added was 0.0025 uCi/mL.
3. The next day the media in which the myotubes had incubated, were transferred to separate vials.
4. The wells were washed 1 to 12 times with DMEM (37°C). Three and three wells were washed the same number of times, so the first 12 well plate had 1 to 4 washes, the next 5 to 8 washes and the last 9 to 12 washes.
5. The media were removed from each well approximately 20 seconds after addition.
6. The cells were given 1 mL DMEM and were incubated in 37 °C, in 5% CO<sub>2</sub> for 24 hours and the media were then transferred to vials.
7. The cells were given 1 mL 2 % Triton X-100 and incubated in 37 °C, in 5% CO<sub>2</sub> for 5 minutes. The wells were scraped thoroughly by using a small rubber scraper.
8. The lysates were transferred to vials.
9. The radioactivity in the media and lysate were measured in Wallac Microbeta Trilux.

### ***Experiment 1b***

#### **Protocol 1b**

1. Myotubes were seeded and differentiated in a 12 well plate as described above.
2. After the myotubes were differentiated for four days three of wells were added 1 mL radio labelled medium and incubated in 37 ° C, in 5 % CO<sub>2</sub> for 24 hours. The concentration of <sup>14</sup>C-BSA in the medium (M3) added was 0.086 uCi/mL.

3. The next day the media, which the myotubes had incubated in, were transferred to separate vials.
4. Each of the three wells was washed nine times with 1 mL DMEM. Media was added and removed after approximately 20 seconds and transferred to different vials. The entire medium used by washing the cells was collected.
5. The cells were given 1 mL 2 % Triton X-100 and incubated in 37° C for 5 minutes, the wells were scraped thoroughly by using a small rubber scraper.
6. The lysate were transferred to vials.
7. The radioactivity in the media and lysate were measured in Wallac Microbeta Trilux.

## ***Experiment 2***

### **Measuring Lactate dehydrogenase (LDH) activity in the media from myotubes being washed**

#### **Protocol 2a**

1. Human satellite cells were seeded out on a 12-well plate as described above. Nine of the 12 wells were used.
2. After the myotubes were differentiated for 5 days they were washed by adding and removing 1 mL DMEM 3, 6 or 9 times. The wash solutions were collected in different vials. Three wells got the same exposure.
3. Thereafter the cells were incubated in 1 mL DMEM for 24 hours.
4. The day after the media were transferred to eppendorf vials and centrifuged on 3000 rpm for 5 min.

5. The myotubes were given 1 mL 2 % Triton X-100, incubated at 37° C in 5% CO<sub>2</sub> for 5 minutes and the wells were scraped thoroughly by using a small rubber scraper.
6. LDH was measured (as described above) in the media and related to that of total LDH released after complete cell lysis

### Protocol 2b

The next experiment we did was the same as above with a few modifications.

1. Human satellite cells were seeded out on one 12-wells plate (as described above).
2. After five days differentiation the wells were washed with 1 mL DMEM 0, 3, 6 or 9 times.
3. The myotubes were given 0.5 mL DMEM and incubated 24 hours.
4. The myotubes were visually investigated under microscope.
5. The media were centrifuged on 3000 rpm for 5 minutes.
6. The amount of LDH was measured in the supernatant. The myotubes were given 0.4 mL 2 % Triton X-100 and incubated at 37 ° C for 5 minutes, and the wells were scraped thoroughly by using a small rubber scraper. The cell lysate were transferred to vials and LDH and protein were measured.
7. LDH was measured in the media and related to that of total LDH released after complete cell lysis

### *Experiment 3*

**Measure LDH in the media from myotubes with different differentiation status and incubated in media without serum for different periods of time.**

### Protocol 3

1. Human satellite cells were seeded in three 12 well plates as described above.
2. The muscle cells were differentiated for 0, 2 and 5 days and washed two times by adding and removing 1 mL DMEM.
3. The muscle cells were added 450 ul DMEM and incubated in 6 or 18 hours.
4. The media was removed and centrifuged for 5 minutes by 3000 rpm.
5. The wells were added 1 mL PBS, the PBS was removed and the myotubes were lysated in 450 ul 2% Triton X-100. The wells were scraped thoroughly by using a small rubber scraper.
6. LDH in the supernatant was measured and related to the LDH measured in the cell lysis.

### *Experiment 4*

#### **IL-6 concentrations in the media from myotubes with different differentiation status and incubated in media without serum for different periods of time**

Frozen supernatants from experiment 3 were thawed and IL-6 concentrations were measured using commercially available solid phase, high-sensitivity enzyme-linked immunosorbent assay (ELISA) (as described above).

### Experiment 5

#### **IL-6 concentration in media from myotubes incubated 6 hours with and without BFA**

### Protocol 5

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1. Human satellite cells were seeded out in two 12 well plates as described above.
  2. Five days differentiated myotubes were washed six times with 1 mL DMEM
  3. 400 ul DMEM or 400 ul DMEM with 5ug/mL BFA were added.
  4. The myotubes incubated in 0, 3 and 6 hours and the media were removed and centrifuged by 3000 rpm for 5 minutes.
  5. The myotubes were added 100 ul 2 % Triton X-100 and incubated in 37 ° C for 5 minutes.
  6. IL-6 concentrations were measured in the supernatants
  7. LDH amounts were measured in the supernatants and lysates.

### ***Experiment 6***

#### **IL-6 concentration measured in media from myotubes incubated 6 hours in different concentration of BFA**

##### **Protocol 6**

1. Human satellite cells were seeded out in a 12 well plate as described above.
2. Myotubes differentiated five days were washed 6 times with 1 mL DMEM and incubated 6 hours in 400 ul with different concentrations of BFA (1-5 ug/mL)
3. The media got centrifuged at 3000 rpm for 5 minutes
4. Concentrations of IL-6 were measured in the supernatant derived from myotubes incubated with the different concentrations of BFA.
5. The myotubes were added 100 ul 2 % Triton X-100 and incubated in 37 ° C for 5 minutes.
6. LDH activities were measured in the supernatants and lysates

### ***Experiment 7***

#### **Myostatin concentration in media from myotubes incubating 6 and 18 hours without serum**

Frozen supernatants from experiment 3 were thawed and myostatin concentrations were measured by RD 193058100 Human Myostatin ELISA. Samples of each supernatant were taken out and myostatin concentration determined.

### ***Experiment 8***

#### **IL-6 concentration in the supernatant when the myotubes incubated different periods with no serum**

##### **Protocol 8**

1. Human satellite cells were seeded out in two 12 well plates as described above.
2. Myotubes differentiated five days were washed 6 times with 1 mL DMEM and incubated different periods of time; 0, 1, 3, 6, 9, 24, 48 and 72 hours in 400 ul DMEM
3. The media were removed to different vials after incubating and the vials were centrifuged by 3000 rpm for 5 minutes.
4. 200 ul supernatant were transferred to 96 well plate and IL-6 was measured
5. 25 ul supernatant were transferred to 96 well plate and LDH was measured
6. Each of the wells was added 100 ul 2% Triton X-100, incubated 5 minutes in 37 ° C for 5 min.
7. 20 ul of each lysate were transferred to the 96 well plate which is used for LDH measurement.



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8. 50 ul of each lysate were transferred to another 96 well plate and the protein concentrations were determined.

### *Experiment 9*

#### **IL-6 concentration in the media from myotubes incubated in medium with high and low glucose concentration and with or without BFA.**

##### Protocol 9

1. Human satellite cells were seeded out in two 12 well plates as described above.
2. Myotubes were differentiated for five days
3. The wells were washed six times with 1 mL DMEM.
4. The myotubes were incubated for six hours in 400 ul medium with 1 mM, 5 mM and 20 mM glucose, and half of the wells were given insulin. And half of these six different treatments were given 1 ug/mL BFA.
5. After 6 hours the medium was removed, centrifuged and IL-6-concentration was measured on the supernatant.
6. The myotubes were given 2% Triton X-100, incubated in 37°C, in 5 % CO<sub>2</sub> for 5 min.
7. The amount of protein was measured on the cell lysate.

### *Experiment 10*

#### **Incubation with high and low glucose with and without BFA**

##### Protocol 10

1. The myotubes were seeded out on 12 well plates as described above

2. Two days differentiated myotubes were preincubated with either 5 mM or 20 mM glucose for four days.
3. The myotubes were washed 6 times with 1 mL DMEM.
4. The myotubes incubated in 400 ul DMEM or DMEM with 1 ug/mL BFA for 6 hours.
5. The media were transferred to vials centrifuged by 33300 rpm for 40 minutes in 10° C.
6. The supernatants were frozen in - 20°C and sent to Aker University Hospital in dry ice for proteomic analyse.

#### Proteomics at Aker University Hospital

The proteins from supernatants from donor 29 and 37 incubated with BFA were concentrated from 2 mL to 60 ul and 50 ul, respectively. The upconcentration of supernatants from myotubes incubated without BFA resulted in higher final volume after the concentration with the same cut-off value, because these supernatants contained higher concentration of protein. 2 mL of the supernatants derived from myotubes incubated without BFA ended up with 100 ul from both ID 29 and 37. This was not adjusted before the electrophoresis was performed. The samples were concentrated by centrifugation through membranes with a cut-off of 10 kDa. SDS-PAGE and two-dimensional electrophoresis were performed at Hormonlaboratoiet at Aker University Hospital.

Proteins were separated according to their molecular weight in SDS-PAGE using NuPAGE 4-12% Bis-Tris gels (Invitrogen). In two-dimensional electrophoresis the proteins were first separated by isoelectric focusing using Immobiline DryStrip pH 3-10. 7 cm (GE Healthcare) which was rehydrated in 7M urea, 2M thiourea, 4 % CHAPS, 0.2% Pharmalytes and 1.5 mg/mL DTT. The samples (10 ul) were added

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rehydration buffer (115  $\mu$ l) and applied along the whole strip and rehydrated over night. The focusing was run with a Bio-Rad Protean IEF Cell (Bio-Rad). After the focusing the strips were equilibrated in LDS sample buffer (Invitrogen) containing reducing and alkylating solutions and the proteins were applied to SDS-PAGE as described above. Proteins were visualized by staining either with Coomassie or silver. Magic Mark protein standard (Invitrogen) was used to estimate molecular weights. The proteins from the gel were further characterized by Matrix-assisted laser desorption/ionization-time of flight mass spectrometer (MALDI-TOF).

### *Experiment 11*

#### Protocol

1. Satellite cells from donor 29 were seeded out in two 12 well plates and donor 30 and 37 were seeded out in four 175  $\text{cm}^2$  flasks.
2. Myotubes differentiated five days were washed six times with 1 mL DMEM in each well for the 12 well plate and 15 mL for the 225  $\text{cm}^2$  flasks.
3. The wells were added 0.4 mL DMEM or DMEM with 1  $\mu\text{g/mL}$  BFA, and the flasks were added 15 mL DMEM or DMEM with 1  $\mu\text{g/mL}$  BFA and incubated for six hours.
4. The medium were centrifuged for 5 minutes on 4000 rpm.
5. Cytokines in the conditioned media were analysed by Luminex multiplexing technology.
6. The concentration of protein in the lysate was measured.

### 3. Results

Our aim was to identify novel myokines in growth media conditioned by human myotubes. First, the myotube culture conditions had to be determined. Second, we validated our cell model with respect to myokine secretion by measuring IL-6, a previously identified myokine. Finally, we collected myotube conditioned media which were analyzed using a proteomics approach.

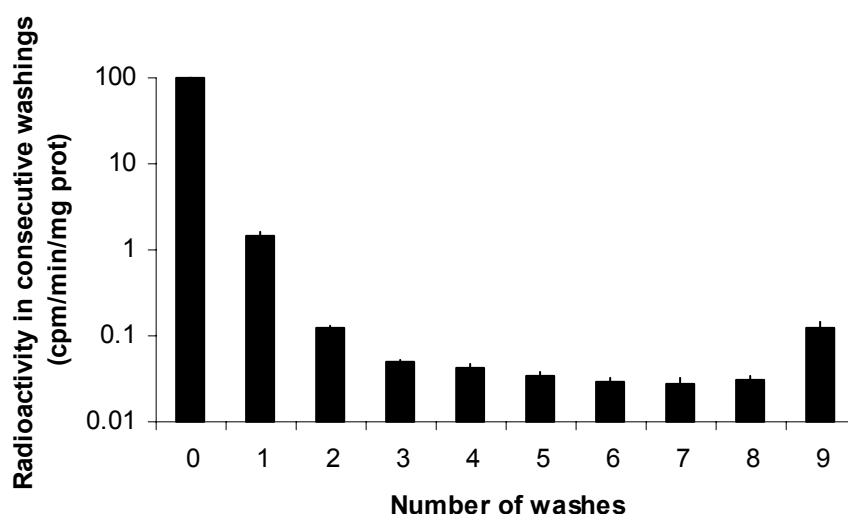
#### 3.1 Eliminating FCS in media by pre-washing cells

Human myotubes are normally cultured in the presence of FCS. For the purpose of proteomics, we needed to generate conditioned media containing proteins derived from myotubes alone, without FCS. When switching from media containing FCS to media without FCS, the cells need to be washed by repetitively adding and removing media (DMEM).

We measured how many times the cells needed to be washed prior to conditioning of the media to diminish the background noise from FCS proteins (mainly BSA) during 2D-gel electrophoresis. The washing procedure would also need to be gentle enough to avoid stress and cell death. To find out how many times we should wash the myotubes to remove appropriate amount of serum proteins we used radiolabelled  $^{14}\text{C}$ -BSA as a marker for BSA and other macroproteins in media. Myotubes were first incubated with  $^{14}\text{C}$ -BSA and then washed 9 times in the media. The washing solutions were collected and their levels of radioactivity were measured (Figure 1).

After removal from the cells, the media in which the myotubes had been initially incubated contained 98 % of the total  $^{14}\text{C}$ -BSA (wash # 0). The radioactivity measured in the first and second wash solution contained 1.5 and 0.1 % of the total radioactivity, respectively (Figure 1, logarithmic scale). Washing solution number 3

to 9 contained  $< 0.05$  % of the total  $^{14}\text{C}$ -BSA (Figure 1).

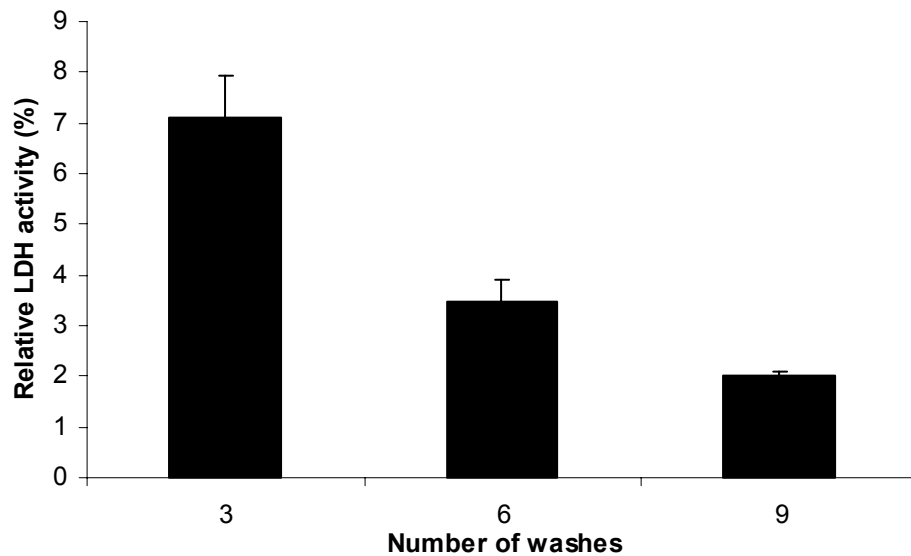


**Figure 1. Amount of  $^{14}\text{C}$ -BSA removed from cultured human myotubes during washing.** After 4 days of differentiation human myotubes were incubated with  $^{14}\text{C}$ -BSA and then washed 9 times. The diagram shows radioactivity, as a measure of  $^{14}\text{C}$ -BSA, in washing solutions # 1-9 relative to the total in all 9 washing solutions. The experiment was performed in triplicates and each point represents mean  $\pm$  SEM. The Figure is representative of two similar experiments.

In our search for new secretory proteins from myotubes it was important to monitor what condition the cells were in. Damaged or dead myotubes might contaminate the conditioned media with intracellular proteins. LDH is an intracellular enzyme, which leaks out of the cell when the membrane is damaged. To explore how washing influenced plasma membrane integrity, we measured LDH activity in the media.

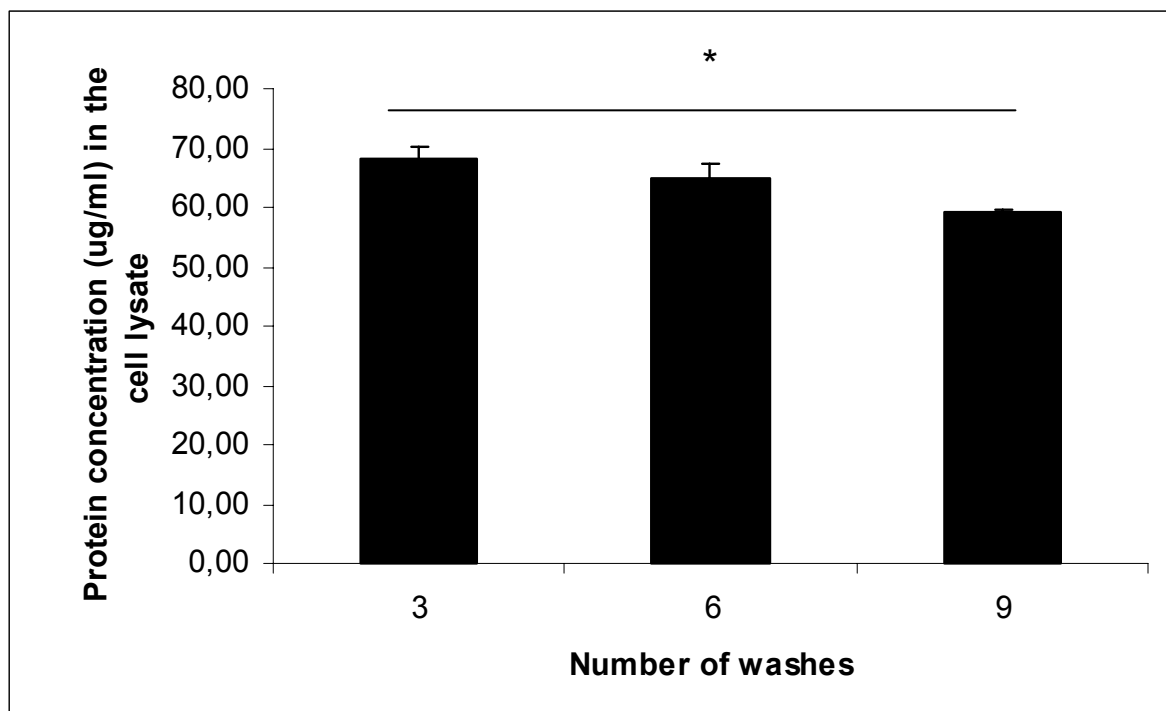
Human myotubes were washed by adding and removing washing solution 3, 6 or 9 times. After this rinsing, the myotubes were cultured for another 24 hours and LDH activity was measured (materials and methods, experiment 3 and Figure 2).

Relative LDH activity was 7.0 %, 3.5 % and 2.0 % after 3, 6 and 9 washes, respectively (Figure 2). Increasing the number of washes from 3 to 9 reduced LDH activity significantly ( $P = 0.03$ ).



**Figure 2. LDH activity in supernatants from myotubes exposed for three levels of washing.** Human myotubes were washed by adding and removing washing solution 3, 6 or 9 times and incubated in medium (DMEM) for 24 hours. The conditioned media were collected and the diagram shows the LDH activity measured in the media related to the total LDH activity measured in lysed cells (relative LDH activity). The experiment was done once in triplicates and each point represents mean  $\pm$  SEM.

Because LDH activity was expected to rise with increasing number of washes we measured an additional variable to see if cells had been lost during the washing procedures. Protein content in cell lysates is an indicator of the amount of cells. Therefore, we compared the amount of total cellular proteins in cells after different washing exposure.



**Figure 3. Protein concentration in the lysate of the myotubes after exposure of different levels of washing.** The myotubes were washed 3, 6 and 9 times with washing solution and incubated for 24 hours. The bars represent protein concentration measured in total cell lysates (mean  $\pm$  SEM) of one experiment performed in triplicates.

The protein concentrations measured in the lysates after exposure of 3, 6 or 9 washes were 68.0, 64.9 and 59.1 ug/mL, respectively (Fig 3). Lysates from myotubes exposed to 9 washes had statistically less protein content than lysates from myotubes exposed to 3 washes ( $P = 0.037$ ).

We also observed in the microscope that increasing the number of washes tended to reduce the number of cells attached to the culturing surface.

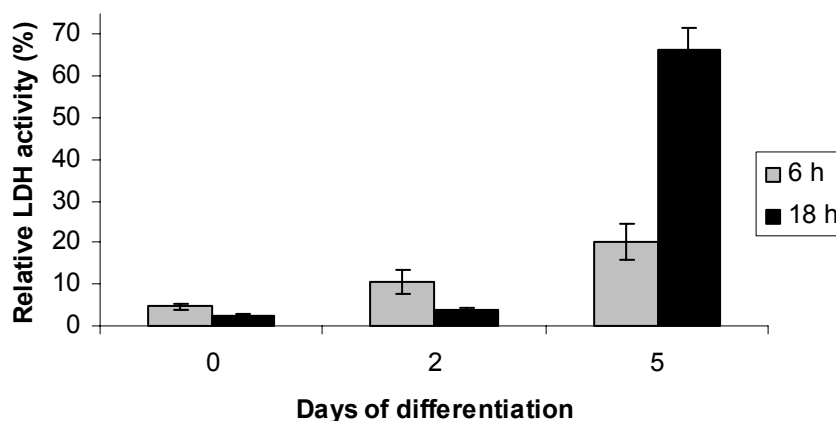
### 3.2 Optimal incubation with FCS-free media

The length of incubation under serum-free conditions is a trade-off between two time-dependent processes: 1) maintenance of cell integrity and 2) reaching detectable

levels of secretory proteins in the conditioned medium. We therefore monitored these two parameters at different time-points.

The medium routinely given cultured human myotubes contains FCS. However, we had to incubate the myotubes in protein-free conditions to avoid detection of proteins derived from FCS during proteomic analysis. We had to find out how the myotubes were influenced by serum-free conditions over time.

We incubated undifferentiated muscle cells and myotubes differentiated for 2 and 5 days in medium with no serum for 6 or 18 hours. To evaluate how incubating 6 and 18 hours with no serum influenced plasma membrane integrity, relative LDH activities were determined (Figure 2).



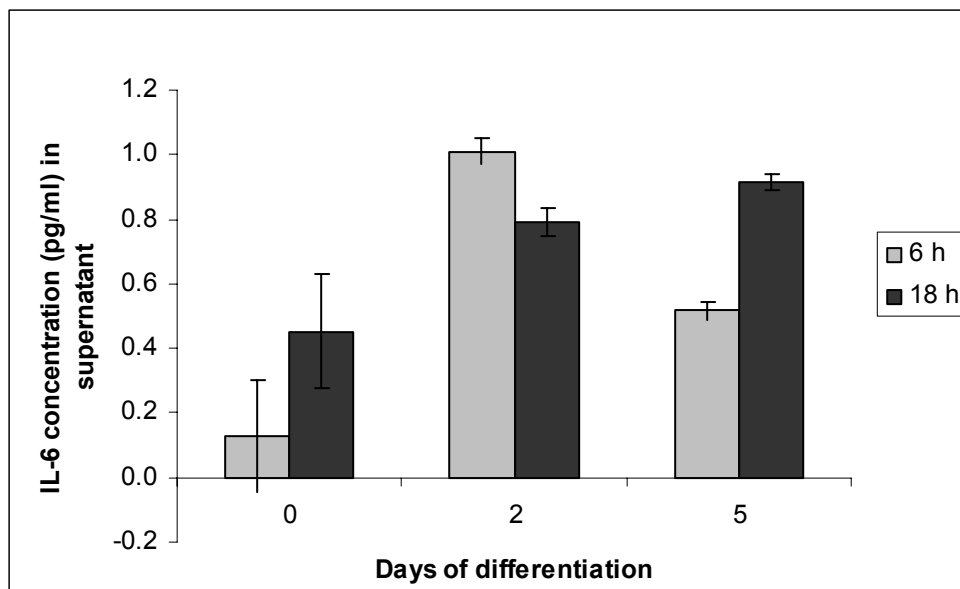
**Figure 4. LDH activity in media from myotubes incubated 6 or 18 hours without FCS.** Myotubes differentiated 0, 2 or 5 days were incubated in medium with no serum for 6 or 18 hours. The bars show mean relative LDH activity  $\pm$  SEM (n=5).

The relative LDH activity in the media was three times higher after 18 hours compared to 6 for myotubes differentiated 5 days.



Myotubes differentiated 0 and 2 days had in general less relative LDH activity than myotubes differentiated 5 days (Figure 4).

Several studies indicate that IL-6 is secreted from myotubes (14;18). Therefore, we chose to measure IL-6 in our media for validation purposes. The aim was to examine if IL-6 could be found in media of our cultured myoblasts and myotubes.



**Figure 5. IL-6 concentration measured in the media from myotubes incubated in medium without FCF**  
IL-6 concentration measured in the media from muscle cells differentiated 0, 2 and 5 days and incubated in medium without serum for 6 or 18 hours. The bars show mean concentration of IL-6 and standard deviation for duplicates from each well (n = 2).

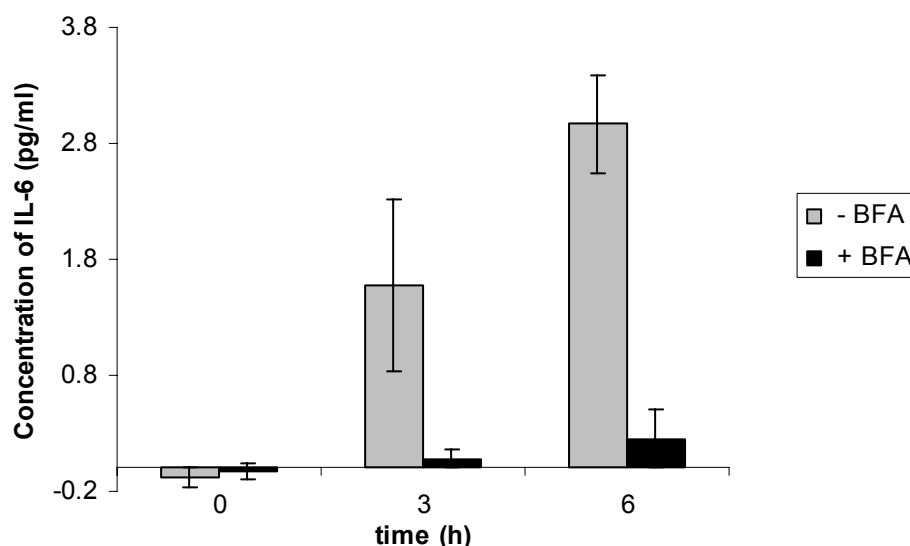
The concentrations of IL-6 in the media of undifferentiated myoblasts were 0.13 pg/mL and 0.45 pg/mL for 6 and 18 hours incubation period, respectively.

Myoblasts differentiated 0 days and myotubes differentiated 5 days had 3.5 and 1.8 times, respectively, higher concentration of IL-6 in the supernatant derived from myotubes incubated 18 hours compared with 6 hours. The medium from myotubes differentiated 2 days and incubated for 6 hours had 1.0 pg/mL IL-6 which was the highest concentration of IL-6 measured in this experiment (Figure 5).

For undifferentiated cells and for cells differentiated in two days, the relative LDH activities were below 10 % for both 6 and 18 hours incubation. For 5 days differentiated myotubes the relative LDH activity after 6 hours incubation was 20.1 % and for cells incubated 18 hours the percentage increased to 66.4 (Figure 4).

### 3.3 Effect of BFA on IL-6 release

We wanted to study proteins secreted, and not released due to damage, from myotubes. BFA is involved in intracellular protein transport and could possible be used as a negative control to investigate secretion of proteins from myotubes. This experiment (materials and methods, experiment 5) was performed to explore this. Myotubes were incubated with and without BFA and the concentrations of IL-6 were measured in the media. IL-6 was thus used as a marker for protein secretion, because we assumed that if BFA inhibited secretion of IL-6 it would most probably inhibit secretion of other proteins as well.



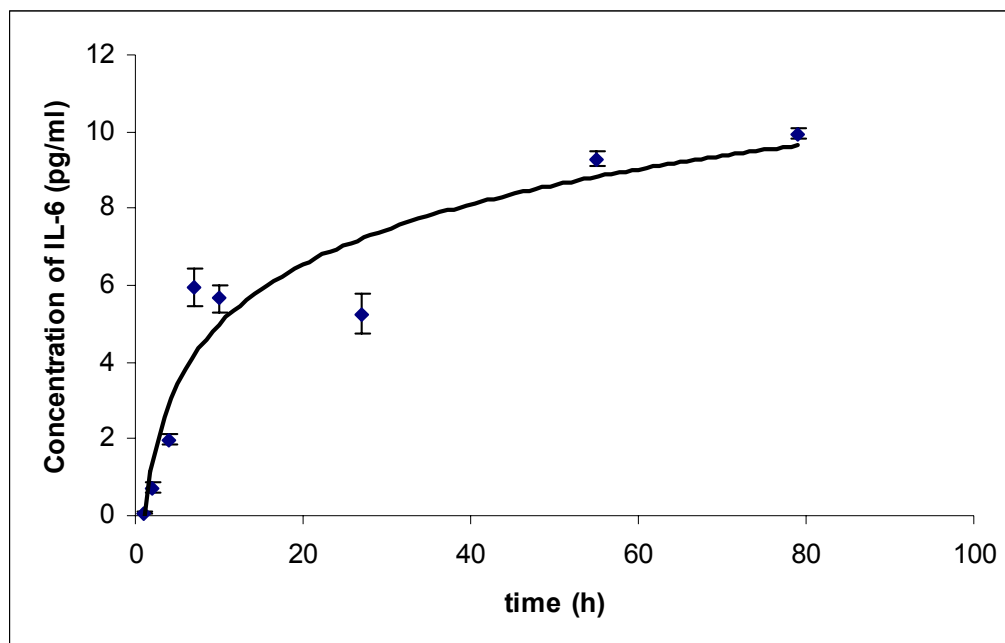
**Figure 6. IL-6 concentration measured in the supernatants from myotubes in media with or without BFA (5 ug/ml).** The bars show mean and SEM from two wells in duplicate (n = 2).

The mean concentration of IL-6 from myotubes incubated in medium without BFA was 19 times higher than the concentration derived from myotubes incubated in medium with BFA, when incubating 3 hours. Myotubes incubated in 6 hours without BFA had 11 times higher concentration of IL-6 in the media compared to myotubes incubated in media with BFA (Figure 6).

The lower concentration of IL-6 in the supernatants from myotubes incubated in media with BFA compared to the incubation in media without BFA probably indicates inhibition of secretion because of BFA.

### 3.4 IL-6 secretion over time

Myotubes were incubated in medium with no serum; 0- 72 hours. The amount of IL-6 was measured in media.



**Figure 6. IL-6 concentration measured in media from myotubes incubated up to 72 hours in DMEM.**

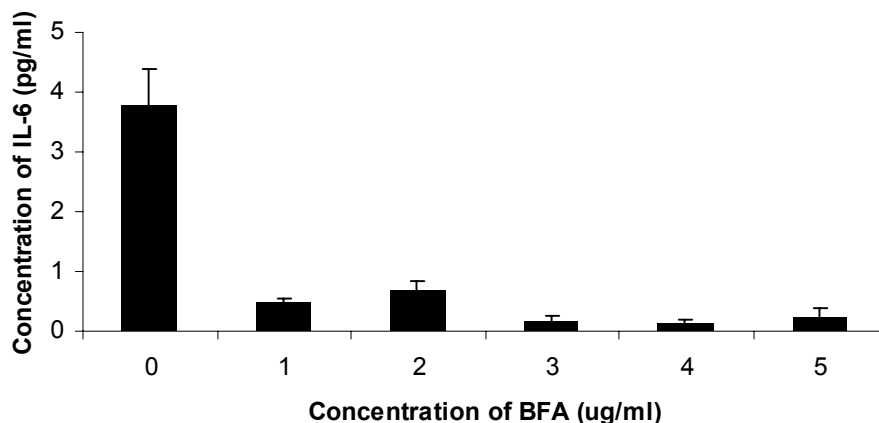
The spots show mean  $\pm$  SEM (n=6) concentrations of IL-6 in the media after the different incubation periods for the duplicates for three wells.

The IL-6 curve was nearly linear the first 6 hours of incubation (Figure 6). The IL-6 concentration in media after 6 hours was 59.8 % of the IL-6 concentration after 72 hours.

The LDH activity increased above twofold from 24 hours incubation to 48 hours incubation (Data not shown).

### 3.5 Optimal concentration of BFA

The experiment above indicated an extensive reduction of IL-6 concentration in conditioned media by 5 ug/mL BFA. Thus, we explored how different concentrations of BFA would influence the concentration of IL-6 in the media. Myotubes were incubated 6 hours with different concentrations of BFA; 1-5 ug/mL. IL-6 concentrations were measured in the supernatants (materials and methods, experiment 6).



**Figure 7. IL-6 measured in medium from myotubes incubated with different concentrations of BFA.**

The five days differentiated myotubes incubated 6 hours with different concentrations of BFA. IL-6 concentrations were measured in the supernatants. The bars show mean concentration ± SEM (n=4) for the concentrations of IL-6 in the media from two wells in duplicate.

The experiment indicated that the lowest concentration of BFA, 1 ug/mL inhibited secretion by 87.5 % (Figure 7).

### 3.6 Myostatin release

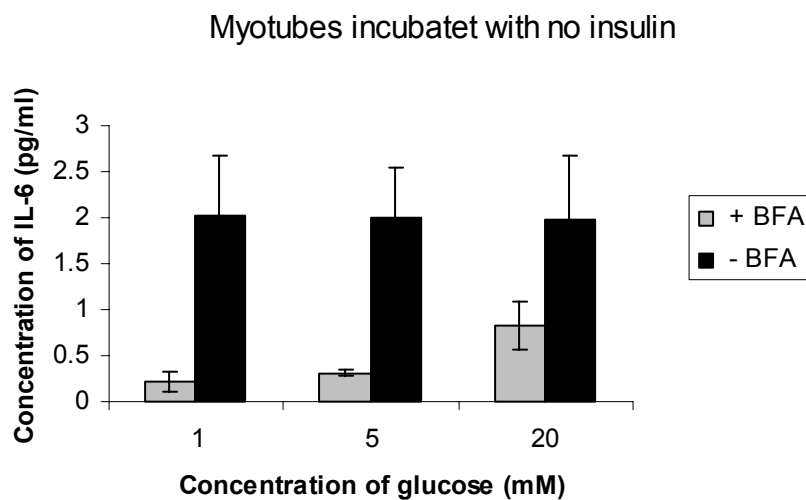
Frozen supernatant from the experiment above (materials and methods, experiment 3) were thawed and a sample of each supernatant was taken out and myostatin concentration determined by ELISA. Myostatin concentrations in the myotube conditioned media were below the detection limit (data not shown).

### 3.7 Effect of glucose on IL-6 secretion

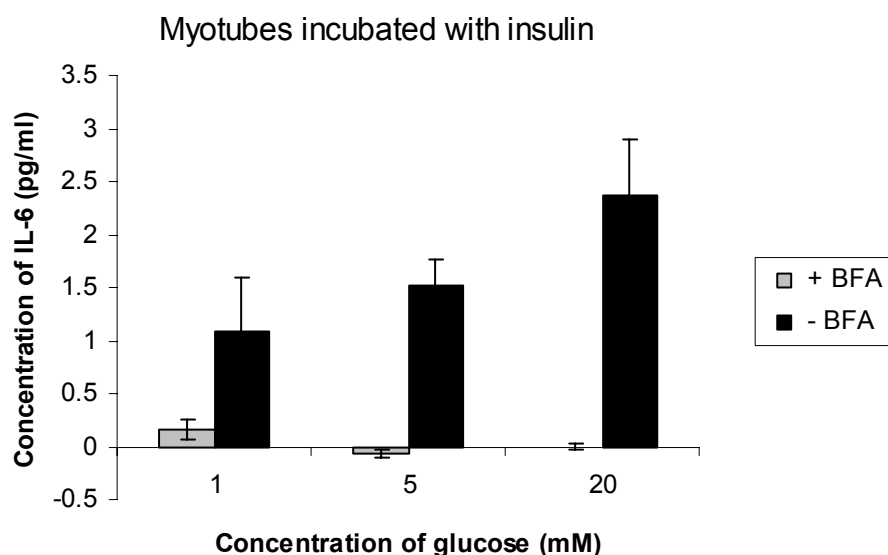
We studied whether incubation of myotubes with metabolic parameters as insulin and glucose, affected the concentration of IL-6 in media.

The myotubes were incubated for 6 hours in medium with three different glucose concentrations, with and without insulin.

BFA is a negative control, indicating if the concentrations of IL-6 in the media are due secretion or leak. The cells were lysed and the amount of protein was measured (materials and methods, experiment 9).



**Figure 8a. IL-6 concentration measured in conditioned media from myotubes incubated with different glucose concentrations.** Myotubes differentiated five days in 12 well plate incubated 6 hours in serumfree media with 1 mM, 5 mM and 20 mM glucose. The bars show mean  $\pm$  SEM (n=4) concentration of IL-6 in the media after six hours incubation with the different exposures from two experiments using media from one well in a duplicate from each experiment.



**Figure 8b. IL-6 concentration measured in media from myotubes incubated with insulin and different concentrations of glucose.** Myotubes differentiated five days in 12 well plates incubated in serumfree media with 1 mM, 5 mM or 20 mM glucose concentrations with insulin for 6 hours. The Figure shows SEM (n=4)

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and mean concentration of IL-6 measured in the media (in duplicate) from two experiments using one well from each experiment (n=4).

IL-6 concentrations in media declined with decreasing glucose concentrations when insulin was present. The release of IL-6 into the media was not affected when the myotubes incubated in the different glucose concentrations without insulin. The glucose effect on IL-6 release in media seems to be insulin dependent (Figure 8a, b).

Incubation of myotubes in different concentrations of glucose (1, 5 and 20 mM) for a 6 hour showed similar effects on IL-6 concentrations in supernatants without insulin (Figure 8b). IL-6 concentrations in the media were reduced with lower glucose concentrations (Figure 8a) in the presence of insulin.

IL-6 concentration in supernatants from cells incubated with insulin and low glucose (1 mM) was 1.1 pg/mL compared to cells incubated with high glucose (20 mM) and insulin was 2.3 pg/mL (Figure 8a).

The media from myotubes incubated with insulin and 5 mM glucose (physiological level), had IL-6 concentration of 1.5 pg/mL (Figure 8a). When the myotubes incubated in the same glucose concentration (5 mM) but without insulin, the concentration of IL-6 in media was 2.0 pg/mL (Figure 8b).

### 3.8 Secretion of cytokines

The medium from myotubes incubated with and without BFA were collected. 13 cytokines in the conditioned media were analysed by Luminex multiplex technology. The concentrations of the cytokines were measured and the total amounts of the cytokines in the media were estimated. The total amount of the cytokines from the well or flask were related to the total amount of protein in the cell lysate from the same well or flask (Table 1). The amount of protein in the cell lysate indicated the amount of cells remaining after washing and incubation in the well or flask. By

relating the amount of cytokines to the amount of protein we adjusted for the eventual differences in myotube density in the different wells or flasks.

The ratio of media volumes (mL) to the growth area (cm<sup>2</sup>) were 0.11 mL/cm<sup>2</sup> for the wells and 0.07 mL/cm<sup>2</sup> for the flasks. The wells had 1.6 times higher volume per cm<sup>2</sup> than the flasks.

**Table 1. The amount of cytokines in the media related to the amount of protein in the cell lysate from the well or flask (pg cytokine/mg protein)**

BFA	Donor 29		Donor 30		Donor 37	
	-	+	-	+	-	+
IL-6	21.36±0.96	1.29±0.08***	2.65±0.25	0.41#±0.14**	200.56±12.18	2.87±0.11***
IL-8	100.22±2.60	2.64±0.30***	4.31±0.027	0.36±0.05***	199.13±4.23	2.15±0.14***
IL-7	9.95±1.16	4.19±1.05*	6.73±0.47	6.39±0.84	5.72±0.05	5.05±0.25
IL-13	10.53±2.15	2.98±0.17*	2.46±0.55	1.93±0.35	1.74±0.00	1.44±0.02***
IL-10	0.15#±0.08	0.25#±0.10	0.21#±0.09	0.42#±0.17	0.47#±0.07	0.16#±0.05
GM-CSF	0.91#±0.13	0.63#±0.09	0.73#±0.13	0.42#±0.08	0.52#±0.05	0.33#±0.06
TNF $\alpha$	ND	ND	ND	ND	ND	ND
IL-1 $\beta$	ND	ND	ND	ND	ND	ND
IFN $\gamma$	ND	ND	ND	ND	ND	ND
IL-12	ND	ND	ND	ND	ND	ND
IL-2	ND	ND	ND	ND	ND	ND
IL-4	ND	ND	ND	ND	ND	ND
IL-5	ND	ND	ND	ND	ND	ND

The mean values  $\pm$  SEM are based on triplicate measurements (except for donor 37 without BFA which are based on duplicates), ND = not detectable, # extrapolated values, \* < 0.05, \*\* < 0.01, \*\*\* < 0.001

IL-6, IL-8, IL-7 and IL-13 were detected in this analysis. IL-6 and IL-8 had also significantly different values in media from myotubes incubated with BFA compared to without.

Supernatant from donor 29 contained the highest concentration of IL-13 per mg protein compared to the other donors. The mean IL-13 concentration in media from myotubes from donor 29 incubated without BFA was 10.5 pg/mL and had more than



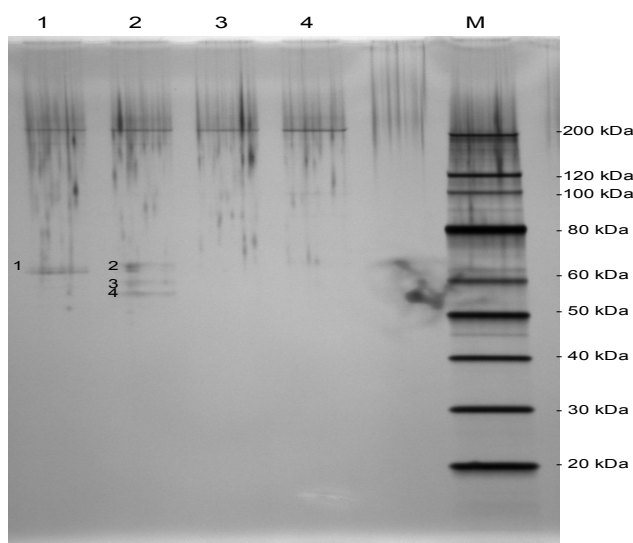
3.5 times higher concentration related to the mean concentration of IL-13 in media from myotubes derived from donor 29 incubated in BFA.

Together with the conditioned media from myotubes, supernatants from hepatocytes and adipocytes were also tested in the same Luminex multiplex analysis. The medium from myotubes was the only medium that showed IL-13 within range of standard curve.

## 3.9 Proteomics of myotubes conditioned media

### 3.9.1 Proteomics of media after preincubation in different glucose concentrations

Myotubes were preincubated in either 5 mM or 20 mM glucose for four days. The myotubes were incubated 6 hours with or without BFA. The supernatants were transferred to vials, frozen and sent to Aker University Hospital for proteomic analysis.

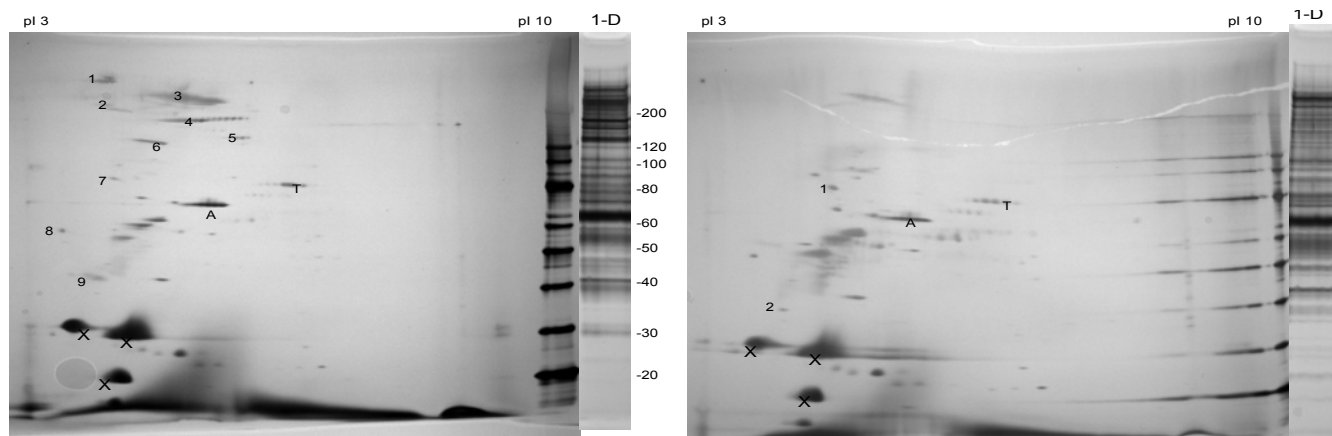


**Figure 9. Silverstrain.** Row 1 and 2 show molecules from myotubes incubated without BFA and row 3 and 4 show molecules from myotubes incubated in BFA. Molecules in row 1 and 3 derive from control (5 mM glucose) and row 2 and 4 derive from myotubes incubated in high glucose (20 mM).

The media from myotubes incubated with BFA (row 3 and 4 Figure 9) gave fewer proteins (bands) than the two others. Compared to the control (row 1, Figure 9) we can see two more bands from myotubes incubated with high glucose (row 2, Figure 9) in the range around 50-60 kDa. These bands were not visible in media from myotubes incubated with BFA. This indicated a secretion inhibition as a consequence of BFA.

### 3.9.2 Proteomics of media from myotubes incubated with and without BFA

Myotubes were incubated 6 hours with or without BFA, and the conditioned medium were analysed by two-dimensional electrophoresis.



**Figure 10. Two-dimensional electrophoresis.** Differentiated myotubes were incubated in media without BFA (left panel) and media with BFA (right panel). The molecules from the conditioned media were first separated by isoelectric focusing and then on size.

Comparing the pictures we can see 9 spots more when the myotubes were incubated without BFA. The media from myotubes incubated without BFA contained high concentrations of protein compared to the media derived from myotubes incubated without BFA. The high protein concentrations lead to clotting of the filters when the concentration of the proteins took place. This resulted in a poorer concentration and a higher final volume with the same cut-off value for media derived from myotubes incubated without BFA. The proteins from media from myotubes from donor 29 and 37 incubated with BFA were concentrated from 2 mL to 60  $\mu$ L and 50  $\mu$ L, respectively. For both donors incubated without BFA 2 mL media ended up with 100  $\mu$ L. Because of no adjustment before the electrophoresis was performed, the gel pictures showed weaker differences between media from myotubes incubated with and without BFA than there really were.

## 4. Discussion

### 4.1 Myotubes used for myokine proteomics

In the present study we explored release of myokines from human skeletal muscle cells in culture. Examination of many different proteins and pathways in cultured muscle cells suggest that, although some differences exist, that these cells contain much of the same proteins and signaling systems as mature muscle fibers. Thus, this model of primary human skeletal muscle cells is a useful and physiological relevant model to use for the study of proteins secreted from human muscle tissue (30), although contractions, which take place *in vivo*, are not present.

Initially it was important to determine the appropriate number of rinses each well should receive to remove a sufficient amount of protein contamination derived from the growth medium (containing 2% serum, FCS), without harming the myotubes growing on the bottom. Reducing background noise, mainly albumin, was necessary before 2-D gel electrophoresis took place. Two experiments using  $^{14}\text{C}$ -labelled-BSA were performed to estimate the amount of protein removed by the washes. Not surprisingly the conclusion was that the more we washed the cells the more proteins were removed.

Our data indicated that the LDH activity in media went down with increasing washes. It is not reasonable that the myotubes would leak less LDH with increasing exposure. One hypothesis was that the washing in addition to eliminating proteins in media, removed weak or damaged muscle cells and that the myotubes remained attached to the culturing surface were more robust and leaked less LDH. That could be the explanation for why the relative LDH activity went down when the washing exposure increased.

Medium containing proteins from FCS is routinely given cultured human myotubes. When assessing how long time the myotubes should incubate without FCS to search

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for new myokines, we first explored the relative LDH activity, and found that it was a 3.3 times higher relative LDH activity for 5 days differentiated myotubes incubated 18 hours compared to 6 hours. When emphasizing cell integrity we concluded that 6 hours is more gently than 18 hours.

Because the concentration of peptides and proteins in the media could be low and difficult to measure we wanted to find the optimal method for collecting media containing as much protein derived from myotubes as possible. To validate our model we measured the known myokine, IL-6. We performed a time course of the IL-6 concentration in the media, which indicated that the release of IL-6 was nearly linear up to 6 hours. We decided to incubate the myotubes for 6 hours also when we searched for other myokines. It is possible that novel myokines will display different release patterns than IL-6, and thus will require longer or perhaps shorter incubations for optimal detection, but after 6 hours incubation we show that at least one myokine is detectable.

## 4.2 Search for novel myokines

### 4.2.1 Immunodetection of myokines involving ELISA

As indicated in previous studies we also detected IL-6 as a protein secreted from human myotubes in culture. We first observed IL-6 in media from the myotubes which was the first sign that our model could be appropriate for detecting myokines. Later we demonstrated the interesting phenomenon that the concentration of IL-6 in media dramatically decreased when the myotubes were incubated in BFA.

Because previous work indicate that IL-6 is released from myotubes and the release is enhanced by low glycogen stores (19), we expected a higher concentration of IL-6 in media from myotubes incubated with low glucose concentration compared to high glucose concentration. We observed the opposite of what we expected; decreased IL-

6 secretion from myotubes incubated in lower glucose concentration when insulin was present.

Insulin seems to facilitate a regulatory role of glucose on the release of IL-6 through a yet unknown mechanism. One could speculate that perhaps during low extracellular glucose concentrations, insulin is able to shunt the intracellular flow of glucose in such a way that the stimuli maintaining basal IL-6 release conditions is reduced.

ChREBP is a transcription factor known to be activated by xylose-5-phosphate (a metabolite in the pentose phosphate branch deriving from glucose-6 phosphate). One hypothesis could be that ChREBP activity is important for IL-6 synthesis and/or release, and is reduced when xylose-5-phosphate levels drop due to the combined effect of low extracellular glucose and high insulin. This however, remains to be elucidated in future experiments.

It is important to remember that a situation where insulin is kept high while the glucose concentration is low, might not be very physiological relevant. As well as the fact that during fasting basal insulin levels never drop to zero, thus future experiments might consider adjusting these parameters within a physiologic range.

#### **4.2.2 Proteomic approach involving 2D-PAGE and MALDI-ToF**

The gel pictures show more spots for the media from myotubes incubated without BFA (Figure 11). This indicates that the BFA inhibits secretion and that our model is appropriate to explore the secretion of proteins from myotubes.

By the method and analyses used for detecting novel myokines, we did not have enough material to identify the proteins visual in the gel-pictures. The challenge for future experiments is to find a way to apply more secretion material to the gel to make it possible further to identify the proteins by MALDI-TOF.

Supernatant from one donor was analysed both by Luminex multiplexing technology and 2D-PAGE. The results from the Luminex analysis showed that the concentration of IL-6 in the supernatant was 80.6 pg/mL. This means that the concentration from 2 mL to 100  $\mu$ L, ends up with the concentration of 1.6 pg IL-6 / $\mu$ L. 10  $\mu$ L and estimated 16.1 pg IL-6 were applied to the gel. This amount was enough to visually detect proteins by 2D-PAGE, but not further to identify the secretion products by MALDI-TOF. We have to add more protein to the gel to exceed the detection limit. When analysing by 2D-PAGE 10  $\mu$ L was applied. We need 300 pg protein to further characterise the protein by MALDI-TOF. In this example we would need 20 times more protein. To achieve these criteria we could try to centrifuge on a higher speed to remove cell compartments, which could improve the concentration of the media by avoiding the filter to plug and end up with a higher concentration of the secretion products. It is also possible to apply a higher volume to the gel. We can also try to use different cut-off values and separate the proteins before applying the gel, to achieve higher concentration of the different proteins.

By choosing 10 as cut-off value we could miss the possible small signal molecules present in media. Future experiments should also search for smaller proteins or peptides.

#### **4.2.3 Immunodetection involving Luminex multiplexing technology**

IL-6 is a known myokine(14;18;28;31). We wanted to find out if we could detect some other known cytokines in the media from the myotubes. We tested 13 different cytokines in the media from myotubes by Luminex multiplexing technology. IL-6, IL-8, IL-7 and IL-13 were detectable.

IL-8 is suggested as a possible myokine in other studies (32;33). IL-8 is a known chemokine that attracts primarily neutrophils. In addition to its chemokine properties IL-8 acts as an angiogenic factor (34). The concentration of IL-8 increases in plasma

in response to exhaustive exercise (32). After 3-hours exercise IL-8 mRNA increased several-fold compared to preexercise (35). IL-8 is also detected in media from myoblasts isolated from biopsies from patients with suspected myopathy (29). In our study we measured IL-8 in media from human myotubes in culture and the concentration decreased dramatically when the myotubes were incubated in BFA (Table 1). This indicates that IL-8 probably is secreted from myotubes, and therefore can be classified as a myokine.

IL-7 is a key cytokine in the immune system, essential for normal development of B cells and T cells. IL-7 is secreted by stromal cells of the red bone marrow and thymus (36). We detected IL-7 in the medium from myotubes in culture (Table 1).

IL-13 is a key inducer of several cytokine-dependent pathologies. Inflammation, mucus production, tissue remodeling, and fibrosis are regulated by IL-13. IL-13 was identified as the primary mediator of liver fibrosis (37). We detected IL-13 in media from myotubes and observed that the concentration decreased after incubation in BFA (Table 1) which indicates that the release possible is due to a secretion.

#### **4.2.4 Concluding remarks**

We have during this work optimized several conditions important to consider when using myotubes as a model for identifying novel myokines. Myotubes have been used as an in vitro model of skeletal muscle for many years, and are therefore well suited for finding, identifying and studying new muscle derived cytokines (myokines). Based on our studies (and previous protocols) 5 days of differentiation was considered sufficient to ensure optimal differentiation of the myoblasts into myotubes.

We conclude from our studies that in the search of novel myokines, human myotubes in culture should be washed 6 times to remove protein from the serum added the media, and the incubation period in medium without serum should be 6



hours to collect medium where we at least know that the concentration of IL-6 is detectable.

The detection of novel myokines by proteomics should be further optimized. Some of the changes could be to centrifuge the media at a higher speed to remove cell particles. Another issue is the problem of up-concentrating the samples as to apply more proteins to the gel. This could be done perhaps by using different filters that can filter larger fractions or by more efficient precipitations techniques. The proteins and peptides could also perhaps be separated by size before application to the gel.

In addition to IL-6 we detected some potentially new myokines which are; IL-7, IL-8 and IL-13. Our studies support the theory that myotubes release proteins with hormonal functions, and that the release perhaps can be influenced by different energy carrying metabolites. Further studies are required to explore the role muscle tissue has in promoting good health and regulating metabolic pathways in the human body.

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